Molecular Cloning of the Gene for a Conserved Major Immunoreactive 28-Kilodalton Protein of Ehrlichia canis: a Potential Serodiagnostic Antigen

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Received 24 August 1998/Returned for modification 23 November 1998/Accepted 14 January 1999

A gene encoding a 28-kDa protein of Ehrlichia canis was cloned, sequenced, and expressed, and a comparative molecular analysis with homologous genes of E. canis, Cowdria ruminantium, and Ehrlichia chaffeensis was performed. The complete gene has an 834-bp open reading frame encoding a protein of 278 amino acids with a predicted molecular mass of 30.5 kDa. An N-terminal signal sequence was identified, suggesting that the protein undergoes posttranslational modification to a mature 27.7-kDa protein (P28). The E. canis p28 gene has significant nucleic acid and amino acid sequence homologies with the E. chaffeensis outer membrane protein-1 (omp-1) gene family, with the Cowdria ruminantium map-1 gene, and with other E. canis 28-kDa-protein genes. Southern blotting revealed the presence of at least two additional homologous p28 gene copies in the E. canis genome, confirming that p28 is a member of a polymorphic multiple-gene family. Amino acid sequence analysis revealed that E. canis P28 has four variable regions, and it shares similar surface-exposed regions, antigenicity, and T-cell motifs with E. chaffeensis P28. The p28 genes from seven different E. canis isolates were identical, indicating that the gene for this major immunoreactive protein is highly conserved. In addition, reactivity of sera from clinical cases of canine ehrlichiosis with the recombinant P28 demonstrated that the recombinant protein may be a reliable serodiagnostic antigen.

Canine ehrlichiosis, also known as canine tropical pancytopenia, is a tick-borne rickettsial disease of dogs that was first described in Africa in 1935 and in the United States in 1963 (9, 10). The disease received more attention and recognition after an epizootic outbreak occurred in U.S. military dogs during the Vietnam War (29). The etiologic agent of canine ehrlichiosis is Ehrlichia canis, a small, gram-negative, obligate intracellular bacterium that exhibits tropism for mononuclear phagocytes (18) and is transmitted by the brown dog tick, Rhipicephalus sanguineus (11). The progression of canine ehrlichiosis occurs in three phases, acute, subclinical, and chronic. The acute phase is characterized by fever, anorexia, depression, lymphadenopathy, and mild thrombocytopenia (27). Dogs typically recover from the acute phase but become persistently infected carriers of the organism without clinical signs of disease for months or even years (12). A chronic phase characterized by thrombocytopenia, hyperglobulinemia, anorexia, emaciation, and hemorrhage, particularly epistaxis, followed by death develops in some cases (27).

Molecular taxonomic analysis based on the 16S rRNA gene has determined that E. canis and Ehrlichia chaffeensis, the etiologic agent of human monocytotropic ehrlichiosis, are closely related (2, 3, 6, 8). Considerable cross-reactivity of the 64-, 47-, 40-, 30-, 29-, and 23-kDa antigens of E. canis and E. chaffeensis has been reported (5, 6, 22, 23). Analysis of immunoreactive antigens with human and canine convalescent-phase sera by immunoblotting has resulted in the identification of immunodominant proteins of E. canis, including a 29-kDa protein (5). In addition, a 30-kDa protein of E. canis has been described as a major immunodominant antigen recognized early in the immune response and is antigenically distinct from the 30-kDa protein of E. chaffeensis (22, 23). Other immunodominant proteins of E. canis with molecular masses ranging from 20 to 30 kDa have also been identified (4–6, 17).

Recently, cloning and sequencing of a multigene family (omp-1) encoding proteins of 23 to 28 kDa have been described for E. chaffeensis (19). The gene (p28) for the 28-kDa immunodominant outer membrane protein of E. chaffeensis, which is homologous to the Cowdria ruminantium map-1 gene, was cloned, and mice immunized with recombinant P28 were protected against challenge infection with the homologous strain based on PCR analysis of peripheral blood 5 days after challenge (19). Molecular cloning of two similar, but nonidentical, tandemly arranged E. canis 28-kDa-protein genes homologous to the E. chaffeensis omp-1 gene family and the C. ruminantium map-1 gene has also been reported (21).

In this study, we describe the molecular cloning, sequencing, characterization, and expression of the gene (designated p28) for a conserved mature 28-kDa immunoreactive protein of E. canis and the presence of a p28 polymorphic multigene family in E. canis. Comparison with E. chaffeensis and other E. canis 28-kDa-protein genes revealed that this gene has the most amino acid homology with the E. chaffeensis omp-1 multigene family. E. canis P28 is a highly conserved major immunodominant protein, and reactivity of sera from clinical canine ehrlichiosis cases with recombinant P28 suggests that the recombinant P28 may be a reliable serodiagnostic antigen.

MATERIALS AND METHODS

Ehrlichiae and purification. The E. canis Florida strain and isolates Demon, DJ, Jake, and Fuzzy were kindly provided by Edward Breitschwerdt, (College of Veterinary Medicine, North Carolina State University, Raleigh). The E. canis Louisiana strain was kindly provided by Richard E. Corstvet (School of Veterinary Medicine, Louisiana State University, Baton Rouge), and the E. canis Oklahoma strain was kindly provided by Jacqueline Dawson (Centers for Disease Control and Prevention, Atlanta, Ga.). Propagation of ehrlichiae was per-
formed in DH82 cells with Dulbecco modified Eagle medium supplemented with 10% bovine calf serum and 2 mM L-glutamine at 37°C. The intracellular growth in DH82 cells was monitored by the presence of E. canis morulae by using general cytologic staining methods. Cells were harvested when 100% of the cells were infected with ehrlichiae and were then pelleted in a centrifuge at 17,000 × g for 20 min. Cell pellets were disrupted with a Braun-Sonic 2000 sonicator twice at 40 W for 30 s on ice. Ehrlichiae were purified as described previously (30). The lysate was clarified by centrifugation at 100,000 × g for 30 min, and the supernatant was concentrated by centrifugation at 80,000 × g for 1 h. Heavy and light bands containing ehrlichiae were collected, washed with sucrose-phosphate-gluatamate buffer (218 mM sucrose, 3.6 mM KH2PO4, 7.2 mM K2HPO4, 4.9 mM glutamate, pH 7.0), and pelleted by centrifugation.

Nucleic acid preparation. E. canis genomic DNA was prepared by disrupting whole cells by treatment with the RNase-free proteinase K digestion method (per ml as described previously (15). This mixture was incubated for 1 h at 56°C, and the nucleic acids were extracted twice with phenol-chloroform-isamyl alcohol (24:24:1). DNA was pelleted by absolute ethanol precipitation, washed once with 70% ethanol, dried, and resuspended in 10 mM Tris pH 7.5. Plasmid DNA was purified by using a High Pure Plasmid Isolation Kit (Boehringer Mannheim). Genomic PCR amplification products were purified by using a QIAquick PCR Purification Kit (Qiagen, Santa Clarita, Calif.).

PCR amplification of the E. canis p28 gene. The regions of the E. canis p28 gene selected for PCR amplification were chosen based on homology (>90%) over the conserved coding region generated from the Jotun-Hein algorithm for the coding region of the E. chaffeensis p28 and C. ruminantium map-1 genes. Forward primer 793 (5‘-GCAAGGACTTGTCACTACT-3‘) and reverse primer 1330 (5‘-CCCTCGCACTGATGGGCCA-3‘) were designed from the complete sequence of E. chaffeensis p28 and C. ruminantium map-1. Primers 394 (5‘-TCTACTTTGCACTTCCACTATTGT-3‘) and primers 394C (5‘-GAGTAACCAACAGCTCCTGC-3‘) were designed to amplify the 5′ and 3′ end of the E. canis p28 gene. The deduced sequence was deposited with the GenBank accession number AF082748. The primer pair 793 and 1330 corresponded to nucleotides 313 to 332 and 307 to 326, respectively. The corresponding region in E. chaffeensis p28 is residues 206 to 225 and in C. ruminantium is residues 307 to 316. PCR products were analyzed in 1% agarose gels. This amplified PCR product was cloned into the pCR2.1 TOPO TA cloning vector to obtain the desired set of restriction enzyme endonucleases.

Sequencing of unknown 5′ and 3′ regions of the p28 gene. A PCR product corresponding to the 3′ and AP1 (2,000-bp) was unidirectionally sequenced with primer 793C to amplify the unknown 5′ end (nucleotides 710 to 687) were used in conjunction with supplied primer AP1. Amplification with these primers 394 and AP1. The deduced sequence overlapped with the 5′ end of the E. canis p28 gene described in this report are as follows: Jake, AF082744; Louisiana, AF082745; Oklahoma, AF082746; Demon, AF082747; DJ, AF082748; Fuzzy, AF082749; and Florida, AF082750.

RESULTS

PCR amplification, cloning, sequencing, and expression of the E. canis p28 gene. Alignment of nucleic acid sequences from E. chaffeensis p28 and C. ruminantium map-1 by using the Jotun-Hein algorithm produced a consensus sequence with regions of high homology (>90%). These homologous nucleic acid sequences (nucleotides 313 to 332 and 823 to 843 of C. ruminantium map-1 and 326 to 348 of E. chaffeensis p28) were targeted as primer annealing sites for PCR amplification. The PCR amplification of the E. canis and E. chaffeensis p28 genes was accomplished with primers 793 and 1330, resulting in a 518-bp PCR product. The nucleic acid sequence of the E. canis PCR product was obtained by sequencing the product directly with primers 793 and 1330. Analysis of the sequence revealed an open reading frame encoding a protein of 170 amino acids, and comparison of the 518-bp sequence obtained from PCR amplification of E. canis with the DNA sequence of the E. chaffeensis p28 gene revealed homology greater than 70%. Adapter PCR with primers 394 and 793 was performed to obtain the 5′ and 3′ segments of the sequence of the entire gene. Primer 394 produced four PCR products (3, 2, 1, and 0.8 kb), and the 0.8-bp product was sequenced bidirectionally with primers 394 and AP1. The deduced sequence overlapped with the 3′ end of the 518-bp PCR product, which was 12 bp to a termination codon. An additional 625 bp of noncoding sequence at the 3′ end of the p28 gene was also sequenced. Primer 394C was used to amplify the 5′ end of the p28 gene with supplied primer AP1. Amplification with these primers resulted in three PCR products (3, 3, and 2 kb). The 2-kb fragment was sequenced unidirectionally with primer 793C. The sequence provided the putative start codon of the p28 gene and completed the 834-bp open reading frame encoding a protein of 278 amino acids. An additional 144 bp of
FIG. 1. Nucleic acid sequence of the E. canis p28 gene, including adjacent 5' and 3' noncoding sequences. The ATG start codon and TAA termination codon are shown in boldface, and the 23-amino-acid leader signal sequence is underlined.
readable sequence in the 5' noncoding region of the p28 gene was generated. Primers EC28OM-F and EC28OM-R were designed from complementary noncoding regions adjacent to the p28 gene. The PCR product amplified with these primers was sequenced directly with the same primers. The complete DNA sequence for the *E. canis* p28 gene is shown in Fig. 1. The p28 PCR fragment amplified with these primers contained the entire open reading frame and sequence encoding 17 additional amino acids from the 5' noncoding primer region. The gene was directionally subcloned into the pTHoHis expression vector, and *E. coli* (BL21) was transformed with this construct. The expressed P28-thioredoxin fusion protein was insoluble. The expressed protein had an additional 114 amino acids associated with the thioredoxin, 5 amino acids for the enterokinase recognition site, and 32 amino acids from the multiple cloning site and 5' noncoding primer region at the N terminus. Convalescent-phase antisera from an *E. canis*-infected dog recognized the expressed recombinant fusion protein but did not react with the thioredoxin control (Fig. 2).

**Nucleic acid sequence homology.** The nucleic acid sequences of *E. canis* p28 (834 bp) and the *E. chaffeensis* omp-1 family of genes (p28 and omp-1A,-1B,-1C,-1D,-1E, and -1F), including signal sequences, were aligned by using the Clustal method to examine homology between these genes (alignment not shown). Nucleic acid homology was equally conserved (68.9%) between *E. canis* p28 and *E. chaffeensis* p28 and omp-1F. Other putative outer membrane protein genes in the *E. chaffeensis* omp-1 family, i.e., omp-1D (68.2%), omp-1E (66.7%), and

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**FIG. 2.** SDS-polyacrylamide gel electrophoresis of expressed 50-kDa recombinant *E. canis* P28-thioredoxin fusion protein (lane 1, arrow) and 16-kDa thioredoxin control (lane 2, arrow) and corresponding immunoblot of recombinant *E. canis* P28-thioredoxin fusion protein recognized by convalescent-phase *E. canis* canine antiserum (lane 3). The thioredoxin control antigen did not react with the *E. canis* antiserum (not shown). Numbers on the left are molecular masses in kilodaltons.
omp-IC (64.1%), C. ruminantium map-1 (61.8%), and the E. canis 28-kDa-protein 1 gene (60%) and 28-kDa-protein 2 gene (partial) (59.5%), were also homologous to E. canis p28. E. chaffeensis omp-IB had the least nucleic acid homology (45.1%) with E. canis p28.

**Amino acid sequence homology.** Alignment of the predicted amino acid sequences of E. canis P28 and E. chaffeensis P28 revealed amino acid substitutions resulting in four variable regions. Substitutions or deletions in the amino acid sequence and the locations of variable regions of E. canis P28 and the E. chaffeensis OMP-1 family were identified (Fig. 3). Amino acid comparison demonstrated that the E. canis P28 protein had the most homology with OMP-1F (68%) of the E. chaffeensis OMP-1 family, followed by E. chaffeensis P28 (65.5%), OMP-1E (65.1%), OMP-1D (62.9%), and OMP-1C (62.9%), C. ruminantium MAP-1 (59.4%), E. canis 28-kDa protein 1 (55.6%) and 28-kDa protein 2 (partial) (53.6%), and E. chaffeensis OMP-1B (43.2%). The phylogenetic relationships based on amino acid sequences show that E. canis P28 and C. ruminantium MAP-1, E. chaffeensis OMP-1 proteins, and E. canis 28-kDa proteins 1 and 2 (partial) are related (Fig. 4).

**N-terminal signal sequence.** The amino acid sequence analysis revealed that the entire E. canis P28 has a deduced molecular mass of 30.5 kDa. The protein has a predicted N-terminal signal peptide of 23 amino acids (MNCKKILITTAILSMDKVSFS) and the OMP-1 protein family (19, 31). A preferred cleavage site for signal peptidases (SIS; Ser-X-Ser) (20) is found at amino acids 21, 22, and 23. An additional putative cleavage site at amino acid position 25 (MNCKKILITTAILSMDKVSFS) identical to the predicted cleavage site of E. chaffeensis P28 (SFS) was also present and would result in a mature E. canis P28 with a predicted molecular mass of 27.7 kDa. The signal cleavage site of the previously reported E. canis 28-kDa protein 1 is predicted to be at amino acid 30. However, signal sequence analysis predicted that E. canis 28-kDa protein 2 had an uncleavable signal sequence.

**Detection of homologous genomic copies of the E. canis p28 gene.** Genomic Southern blot analysis of E. canis DNA was performed following complete independent digestions with restriction enzymes BanII, EcoRV, HaeII, KpnI, and SpeI, which do not have restriction endonuclease sites in the p28 gene. In addition, digestion with AseI, which has internal restriction endonuclease sites at nucleotides 34, 43, and 656, revealed the presence of at least three homologous p28 gene copies (Fig. 5). Although E. canis p28 has internal AseI restriction sites, the DIG-labeled probe used in the hybridization experiment targeted a region of the gene within a single DNA fragment generated by the AseI digestion of the gene. Digestion of genomic DNA of E. canis with AseI produced three bands (approximately 566 bp, 850 bp, and 3 kb) that hybridized with the p28 DNA probe, indicating the presence of multiple genes homologous to p28 genes in the genome. Digestion with EcoRV and SpeI produced two bands that hybridized with the p28 gene probe.

**Predicted surface probability and immunoreactivity.** Analysis of E. canis P28 by hydrophathy and hydrophilicity profiles predicted surface-exposed regions on P28 (Fig. 6). Eight major surface-exposed regions consisting of three to nine amino acids were identified on E. canis P28 and were similar to the profile of surface-exposed regions on E. chaffeensis P28 (Fig. 6). Five of the larger surface-exposed regions on E. canis P28 were located in the N-terminal region of the protein. Surface-exposed hydrophilic regions were found in all four of the variable regions of E. canis P28. Ten T-cell motifs in E. canis P28 were predicted by using the Rothbard-Taylor algorithm (24), and high antigenicity of P28 was predicted by the Jameson-Wolf antigenicity algorithm (Fig. 5) (13). Similarities in antigenicity
and T-cell motifs were observed between *E. canis* P28 and *E. chaffeensis* P28.

**Homology of p28 gene sequences from different *E. canis* isolates.** The p28 genes from seven *E. canis* isolates, four from North Carolina and one each from Florida, Oklahoma, and Louisiana, were amplified by PCR with primers EC28OM-F and EC28OM-R and sequenced directly with the same primers. Alignment of the p28 gene nucleic acid sequences revealed that the p28 genes from these isolates were identical.

**Serodiagnosis.** Sera from six clinical cases of canine ehrlichiosis were incubated with the recombinant protein in immunoblots at a 1:100 dilution. Antibodies in the sera of five dogs (83%) reacted with the P28 recombinant protein (Fig. 7).

**DISCUSSION**

Proteins with similar molecular masses have been identified and cloned from multiple rickettsial agents, including *E. canis*, *E. chaffeensis*, and *C. ruminantium* (14, 19, 21). In this report, we demonstrated the cloning, expression, and characterization of a gene encoding a mature 28-kDa protein of *E. canis* that is homologous to the omp-1 multiple-gene family of *E. chaffeensis* and the *C. ruminantium* map-1 gene. The *E. canis* p28 gene is also homologous, but different from the previously reported *E. canis* 28-kDa-protein 1 gene (complete) and 28-kDa-protein 2 gene (partial) (21). Previous studies have identified a 30-kDa protein of *E. canis* that reacts with convalescent-phase antisera against *E. chaffeensis*, but this protein was believed to be antigenically distinct (22). Our findings, based on comparison of amino acid substitutions in four variable regions of *E. canis* P28, support this possibility. Together these findings also suggest that the amino acids responsible for the antigenic differences between *E. canis* and *E. chaffeensis* P28 are located in these variable regions and are readily accessible to the immune system. Reddy et al. (21) reported that immunoreactive peptides were located in the variable regions of the 28-kDa proteins of *C. ruminantium*, *E. chaffeensis*, and *E. canis*. Analysis of *E. canis* P28 and *E. chaffeensis* P28 revealed that all of the variable regions have predicted surface-exposed amino acids. A study with dogs demonstrated a lack of cross-protection between *E. canis* and *E. chaffeensis* (7). This observation may be related to antigenic differences in the variable regions of P28 as well as in other immunologically important antigens of these ehrlichial species. Another study found that convalescent-phase human antisera from *E. chaffeensis*-infected patients recognized a 29- or 28-kDa protein(s) of *E. chaffeensis* and also reacted with homologous proteins of *E. canis* (5). Homologous and cross-reactive epitopes on *E. canis* P28 and *E. chaffeensis* P28 appear to be recognized by the immune system.

Several reports have demonstrated that the 30-kDa antigen of *E. canis* exhibits strong immunoreactivity (22, 23). Antibodies in convalescent-phase antisera from humans and dogs have consistently reacted with proteins in this size range from *E. chaffeensis* and *E. canis*, suggesting that they may be important serodiagnostic as well as immunoprotective antigens (5, 6, 22). In addition, antibodies to 30-, 24-, and 21-kDa proteins develop early in the immune response to *E. canis* (22, 23), suggesting that these proteins may be especially important in the immune response during the acute stage of disease and thus...
may be particularly useful for serodiagnosis. In addition, a family of homologous genes encoding outer membrane proteins with molecular masses of 28 kDa have been identified in *E. chaffeensis*, and mice immunized with recombinant *E. chaffeensis* P28 appeared to have developed immunity against homologous challenge (19). The P28 of *E. chaffeensis* has been demonstrated to be present in the outer membrane, and immunolectron microscopy has localized the P28 on the surface of the organism, thus suggesting that it may serve as an adhesin (19). It is likely that the P28 of *E. canis* identified in this study has a similar location and function. The immunoprotective capacity of *E. canis* P28 is not known, but similar studies with *E. chaffeensis* P28 suggest that it may be a potential vaccine candidate.

There is evidence that the P28 from *E. canis* may be post-translationally processed from an immature 30-kDa protein to a mature 28-kDa protein. Recently, a signal sequence was identified on *E. chaffeensis* P28 (31), and N-terminal amino acid sequencing has verified that the protein is posttranslationally processed, resulting in cleavage of the signal sequence to produce a mature protein (19). Sequences of OMP-1F and OMP-1E have also been proposed as leader signal peptides (19). Signal sequences identified on *E. chaffeensis* OMP-1F, OMP-1E, and P28 are homologous to the leader sequence of *E. canis* P28. However, two N-terminal signal sequences were identified on *E. canis* P28 within a 5-amino-acid region (SISFS). The first signal sequence produces a leader peptide two amino acids shorter than that observed on the *E. chaffeensis* P28, due to a single amino acid substitution (serine) at position 21. The second signal sequence is identical to those on *E. chaffeensis* P28, OMP-1F, and OMP-1E and produces a leader peptide consisting of 25 amino acids. The homologies of the 25-amino-acid leader signal peptides of *E. chaffeensis* OMP-1F, OMP-1E, and P28 to *E. canis* P28 are 72, 68, and 64%, respectively. N-terminal amino acid sequencing could verify the cleavage site of the signal sequence of *E. canis* P28, but it is likely that the P28 *E. canis* protein that we have cloned is subject to posttranslational modification similar to that observed with *E. chaffeensis* P28.

Comparison of the p28 genes from different strains of *E. canis* revealed that the gene is apparently completely conserved. Studies involving *E. chaffeensis* have demonstrated immunologic and molecular evidence of diversity in the p28 gene. Patients infected with *E. chaffeensis* have variable immunoreactivity to the 29- and 28-kDa proteins, suggesting that there is antigenic diversity (5), and recent molecular evidence has been generated to support antigenic diversity in the p28 gene from *E. chaffeensis* (31). However, differences in the host response to *E. chaffeensis* P28 may also explain some of the observed immunologic variability. A comparison of the p28 genes of five *E. chaffeensis* isolates revealed that two isolates (Sapulpa and St. Vincent) were 100% identical but that three others (Arkansas, Jax, and 91HE17) were divergent by as much as 13.4% at the amino acid level. The conservation of *E. canis* p28
suggests that E. canis strains found in the United States may be genetically identical, and thus E. canis p28 is an attractive vaccine candidate for canine ehrlichiosis in the United States. Further analysis of E. canis isolates outside the United States may provide information regarding the origin and evolution of E. canis. The documented immunoreactivity and conservation of the P28 protein suggests that it may be a reliable serodiagnostic antigen, and this proposal is further supported by the high rate of reactivity of clinical canine ehrlichiosis specimens with p28 in our study.

The presence of multiple polymorphic genes homologous to E. canis P28 corresponds to the presence of similar multiple-gene families in E. chaffeensis and Anaplasma marginale (1, 19). Six genes were found in the omp-1 gene family of E. chaffeensis, and an msp-3 multiple-gene family has been described for A. marginale. In our study, Southern blot hybridization of E. canis genomic DNA (Jake strain) digested with Asel1 and hybridized with a DIG-labeled p28 probe revealed the presence of at least three gene copies that were homologous to the p28 gene. The restriction enzyme Asel1 cuts within the p28 gene; however, the p28 probe was designed to be complementary with sequences internal to the Asel1 restriction sites. In addition, Asel1 cuts within the noncoding region found between the tandemly arranged E. canis 28-kDa-protein genes described previously (21). Thus, the three p28 genes would be found on separate DNA fragments. The largest fragment from the Asel1 digest (3 kb) that hybridized with the p28 probe is at least three times larger than the p28 gene. Therefore, the possibility of additional genes within this 3-kb fragment that are homologous to p28, and different from those already reported, cannot be eliminated. The hybridization pattern does suggest that all p28 gene copies may be tandemly arranged along a single stretch of DNA. The role of multiple homologous genes is not known at this point; however, persistence of E. canis infections in dogs could conceivably be related to antigenic variation due to variable expression of homologous p28 genes, thus enabling E. canis to evade immune surveillance. Variation of msp-3 genes in A. marginale is partially responsible for variation in the MSP-3 protein, resulting in persistent infections (1). In addition, temperature-related gene expression resulting in phenotypic change in Borrelia hermsii has also been reported (26). Studies to examine p28 gene expression by E. canis in healthy and chronically infected dogs would provide insight into the role of the p28 gene family in persistent E. canis infections.

ACKNOWLEDGMENTS

This study was supported by funding from the Clayton Foundation for Research.

We thank Patricia Croquet-Valdes and John Stenos for helpful technical assistance and Josie Ramirez-Kim for expert secretarial assistance with the preparation of the manuscript.

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